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Dual Role of α-Acetolactate Decarboxylase in *Lactococcus lactis* subsp. *lactis*

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The α-acetolactate decarboxylase gene *aldB* is clustered with the genes for the branched-chain amino acids (BCAA) in *Lactococcus lactis* subsp. *lactis*. It can be transcribed with BCAA genes under isoleucine regulation or independently of BCAA synthesis under the control of its own promoter. The product of *aldB* is responsible for leucine sensibility under valine starvation. In the presence of more than 10 μM leucine, the α-acetolactate produced by the biosynthetic acetohydroxy acid synthase IlvBN is transformed to acetoin by AldB and, consequently, is not available for valine synthesis. AldB is also involved in acetoin formation in the 2,3-butanediol pathway, initiated by the catabolic acetolactate synthase, AlsS. The differences in the genetic organization, the expression, and the kinetics parameters of these enzymes between *L. lactis* and *Klebsiella terrigena*, *Bacillus subtilis*, or *Leuconostoc oenos* suggest that this pathway plays a different role in the metabolism in these bacteria. Thus, the α-acetolactate decarboxylase from *L. lactis* plays a dual role in the cell: (i) as key regulator of valine and leucine biosynthesis, by controlling the acetolactate flux by a shift to catabolism; and (ii) as an enzyme catalyzing the second step of the 2,3-butanediol pathway.

Synthesis of the three branched-chain amino acids (BCAA), leucine, isoleucine, and valine, has been studied in detail in organisms as diverse as bacteria, fungi, and plants (for reviews, see references 8, 32, 57, and 58). A particular feature of this synthesis is that it is carried out, in part, by the same enzymes for the three amino acids (Fig. 1). However, the relative amounts of the three amino acids in cell proteins are not the same, since leucine, valine, and isoleucine represent 39, 36, and 25%, respectively, of the total BCAA in *Escherichia coli* (40). Regulation of the BCAA synthesis is therefore necessarily complex. A global regulation model has been established only for *E. coli* (58). Two levels of regulation, concerning gene transcription and enzyme inhibition, have previously been described.

E. coli genes which encode BCAA synthesis enzymes form three clusters and are organized in five transcription units (2). Initiation of transcription of all the genes except *ilvC* is regulated by nonspecific, pleiotropic regulators responding to amino acid starvation and different changes in the medium (58). In addition to this control, a mechanism of transcriptional attenuation dependent on the synthesis of a leader peptide as described first for the tryptophan operon (33) negatively regulates *ilvBN*, *ilvGMEDA*, and *leuABCD*. These mechanisms allow the expression of the enzymes necessary for BCAA synthesis when needed.

Activity of several BCAA-synthesizing enzymes is regulated by retroinhibition (Fig. 1). This control is simple in the case of leucine and isoleucine, since each amino acid inhibits an enzyme specific for an early step of its synthesis (LeuA and IlvA, respectively [53, 57]). In contrast, the first enzyme involved in valine synthesis is also required for the synthesis of the other two BCAA. In *E. coli*, three isoenzymes catalyze this reaction (IlvBN, IlvIH, and IlvGM), and only two are inhibited by valine (58).

Regulation of BCAA synthesis is poorly understood for other organisms. In *Bacillus subtilis*, structural genes form one operon, comprising *ilvBNCleuACBD*, and two genes, *ilvA* and *ilvD*, are independent (5). The operon is regulated by transcriptional attenuation, dependent on leucyl-tRNA synthetase (20). In *Corynebacterium glutamicum*, the transcription level of the *ilvBNC* operon is increased in the presence of ketobutyrate, the product of *ilvA* and the substrate of *ilvBN* (31). Furthermore, for these bacteria as for *E. coli*, LeuA and IlvA were shown to be regulated by retroinhibition in the presence of leucine and isoleucine, respectively (23, 38, 41, 58, 60). Lastly, in *E. coli* and *C. glutamicum*, IlvBN is inhibited by valine (12).

The *Lactococcus lactis* subsp. *lactis* BCAA structural genes are clustered in a single operon (Fig. 2) (16). Regulation of BCAA biosynthesis appears to be mediated by isoleucine and leucine, which inhibit growth of *L. lactis* subsp. *lactis* (17). Isoleucine affects transcription of the operon by repression, and leucine affects transcription by transcriptional attenuation (16, 19). However, regulation by leucine appears also to involve a deviation of the metabolic flux from BCAA synthesis towards acetoin (18, 42, 45).

Acetoin fermentation occurs in different bacteria in response to environmental changes, such as oxygen concentration, low pH, or pyruvate excess. The first step of this fermentation, acetolactate (AL) synthesis by AL synthase, is similar to that carried out by the acetohydroxy acid synthase (AHAS) in BCAA synthesis (Fig. 1), but the expression of AL synthase is controlled independently of amino acid biosynthesis genes in *L. lactis*, *B. subtilis*, and *Klebsiella terrigena*. AL is transformed to acetoin by AL decarboxylase (ALDC) or converted sponta-

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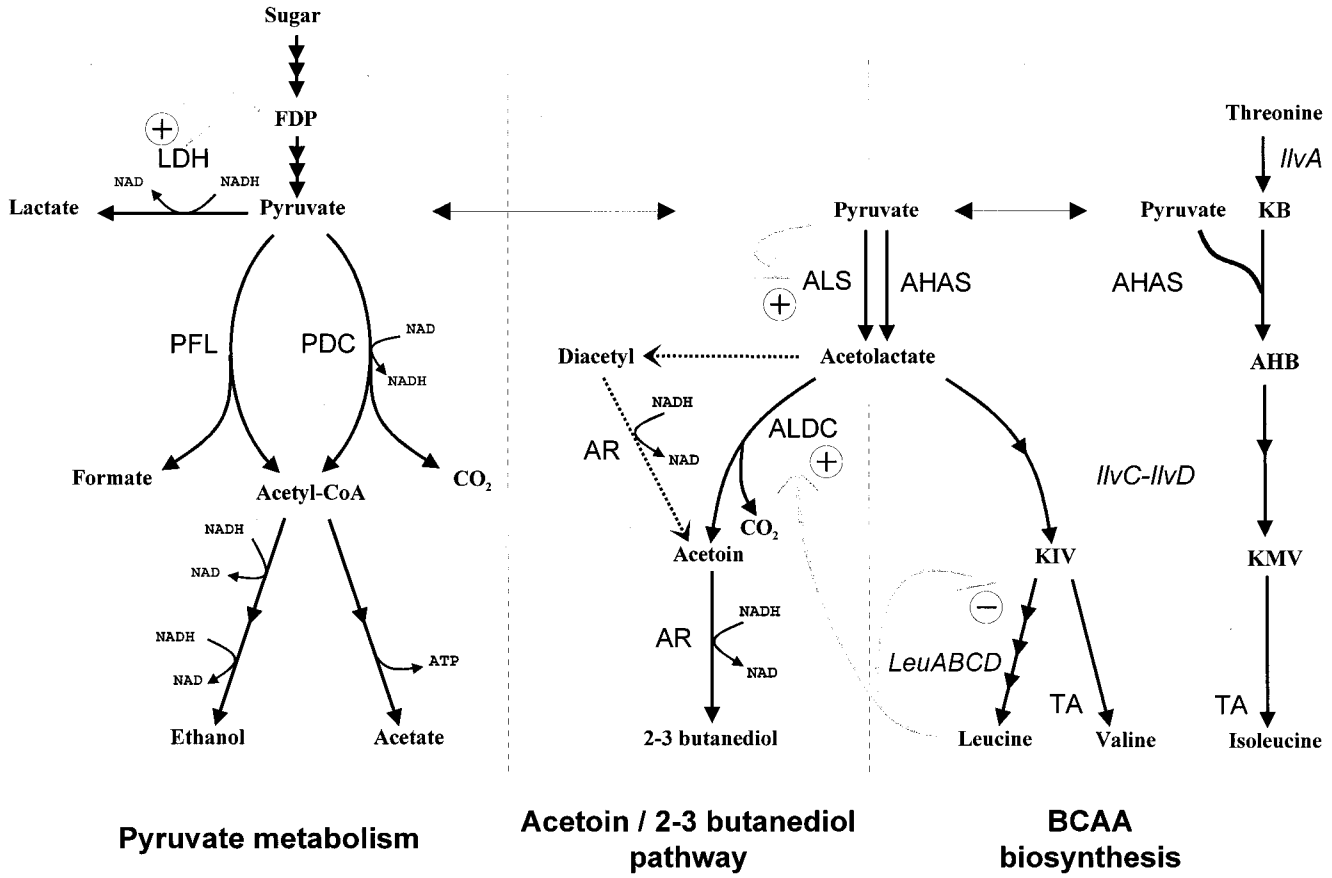

FIG. 1. Interconnection of pyruvate, 2,3-butanediol, and BCAA metabolisms in *L. lactis*. Black arrows indicate the main enzymatic reactions detected in *L. lactis*. Dotted arrows indicate the alternative pathway to acetoin by chemical decarboxylation of AL. Gray arrows indicate factors activating or biochemically inhibiting the reactions. ALS, catabolic α-acetolactate synthase; AHAS, anabolic α-AHAS, encoded by *ilvBN*; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; PDC, pyruvate dehydrogenase complex; AR, diacetyl-acetoin reductase; TA, transaminase; KIV, ketoisovalerate; KB, ketobutyrate; AHB, acetohydroxybutyrate; KMV, ketomethylvalerate; IlvA, threonine desaminase.

neously to diacetyl in the presence of oxygen. The diacetyl is reduced to acetoin, which is converted to 2,3-butanediol by diacetyl-acetoin reductase.

AL is a central metabolite, involved in both anabolism and catabolism. The control of its partition between the two pathways is thus of particular importance. ALDC, which directs the AL flux towards catabolism, is encoded within the cluster of genes of the acetoin pathway in *K. terrigena* and *B. subtilis*. We show in this paper that the *aldB* gene, encoding ALDC, is within the operon that encodes the genes of the BCAA biosynthesis in *L. lactis* (Fig. 2). This genetic linkage, and the fact that ALDC is allosterically activated by leucine, suggests that ALDC participates in the regulation of BCAA synthesis. We have previously reported that mutants selected in the presence of leucine as an inhibitor of growth were producing high amounts of AL but no acetoin during citrate metabolism in milk (18). These mutants were shown to be defective for ALDC. However, the role of ALDC as regulating the pool of AL in the cell during BCAA metabolism was not demonstrated. Here we report that there is a single ALDC activity in *L. lactis* and that it plays a dual role in regulation of both BCAA biosynthesis and pyruvate catabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are presented in Table 1 and Fig. 2. *L. lactis* subsp. *lactis* NCDO2118 was grown at 30°C in M17 medium (54), in which lactose was replaced by glucose, or in chemically defined medium (CDM) (43), in which BCAA composition was modified as mentioned below. Strains JIM4571 and JIM4572 were obtained by incubating strain NCDO2118 in CDM without valine and isoleucine for 5 days as described previously (18). *E. coli* was grown in Luria-Bertani medium or in the M63 minimal medium (49) at 37°C. When necessary, erythromycin (5 μg/ml for *L. lactis*), ampicillin (50 μg/ml for *E. coli*), or tetracycline (10 μg/ml for *L. lactis* and *E. coli*) was added to the medium.

DNA and RNA manipulations. Plasmid and chromosomal DNA were prepared as previously described (34, 49, 50). *L. lactis* cells were transformed by a standard electroporation procedure (24). Southern blotting, DNA hybridization, and other molecular techniques were performed as described previously (49). DNA probes were prepared by nick translation with (α-^{32}P)dCTP (Amersham) according to the supplier's recommendations (Boehringer Mannheim).

E. coli clones for sequencing were obtained by subcloning specific DNA fragments in the pBluescript plasmid and creating a set of plasmids with nested deletions, by using exonuclease III-mung bean nuclease as specified by the supplier (Stratagene). The DNA sequence was determined by using the *Taq* Dye Primer Cycle sequencing kit and a 370A sequencer (Applied Biosystem, Foster City, Calif.). The reported sequence (Fig. 3) was determined at least twice on both strands. The DNA and protein sequences were analyzed with the GCG software package (Genetics Computer Group, University of Wisconsin).

Chromosomal *ald* genes were mutated by a two-step procedure (6). The plasmids containing the construction were first integrated via a Campbell-like mode of insertion into the chromosome at a restrictive temperature (37.5°C) and then excised at a permissive temperature (28°C). For this purpose, a 4-kb fragment carrying the *tetM* gene from Tn*1545* was inserted in the *Pst*I site within the *aldB* gene or in the *Nsi*I site within the *aldR* gene carried on plasmid pIL557 (Fig. 2). This construction was transferred to the thermosensitive plasmid pVE6004 (35) and introduced into *L. lactis*. The chromosomal structure resulting from the two-step procedure exchange was confirmed by Southern hybridization.

RNA was prepared from cells grown exponentially in CDM supplemented

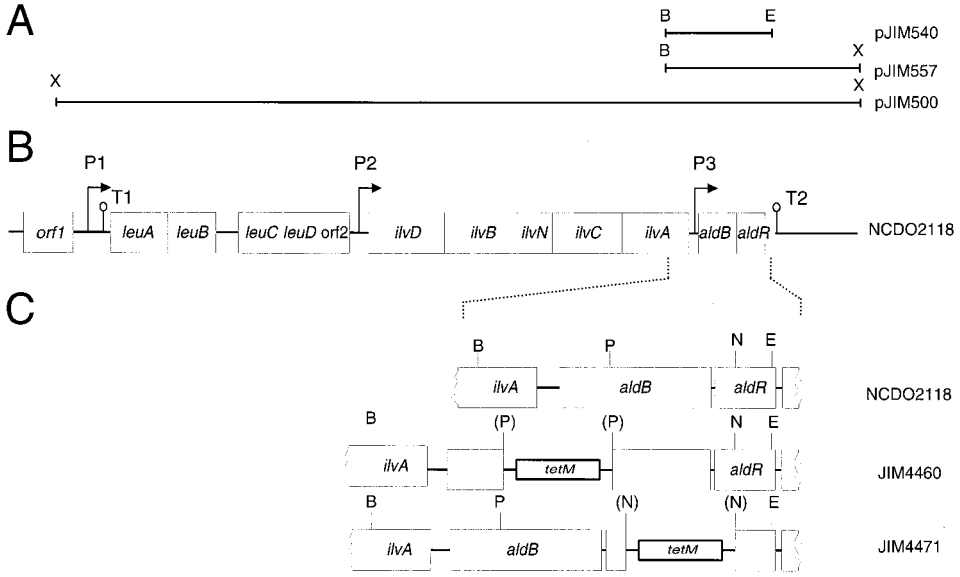

FIG. 2. Organization of the *leu-ilv-ald* cluster and constructs used in this work. (A) Insert of the different plasmids used. (B) Organization of the *leu-ilv-ald* genes. P1, P2, and P3 refer to transcription promoters, and T1 and T2 refer to putative transcription terminators. (C) Gene replacement on the chromosome. The names of the strains and plasmids are on the right. Restriction enzymes: X, *Xba*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; N, *Nsi*I. Parentheses indicate that the site was destroyed by the construction.

with BCAA, washed three times, and transferred to CDM with and without the BCAA for 20 min. Total RNA was prepared as previously described for *B. subtilis* (15). A Bethesda Research Laboratories RNA marker was used to estimate the mRNA size. Northern hybridizations were performed according to the method of Sambrook et al. (49). The 5′ end of mRNA was mapped by primer extension with an oligonucleotide complementary to the region between nucleotides 372 and 390 of the reported sequence. Primer extension products were analyzed on standard sequencing gels.

Determination of fermentation products. AL and the total amount of diacetyl plus acetoin were assayed as previously reported (39, 59, 61). Lactose, glucose, lactate, acetate, formate, ethanol, pyruvate, and butanediol production was determined by high-performance liquid chromatography with an HPX-78P anion-exchange column (Bio-Rad Laboratories, Inc.) with 5 mM H_2SO_4 as the eluent at 48°C.

Nucleotide sequence accession number. For the *L. lactis* NCDO2118 *ald* region, the GenBank nucleotide sequence accession number is U92974.

RESULTS

Analysis of the *ald* genes. We have previously reported the cloning and sequence analysis of an 18.5-kb segment of the *L. lactis* subsp. *lactis* chromosome which carries the genes involved in BCAA synthesis (16). The genes appeared to form an operon, but no typical rho-independent terminator structure was found after the last biosynthetic gene (*ilvA*). We surmised that the operon was longer and determined the sequence of a downstream 1.5-kb region (Fig. 3). Two open reading frames (ORFs) were found in this region followed by a putative terminator. The first ORF encodes a protein of 236 amino acids, which is 31 to 37% identical to ALDC from *B. subtilis*, *Bacillus brevis*, *K. terrigena*, *Enterobacter aerogenes*, *Acetobacter aceti*, and *Coxiella burnetii*, present in the GenBank database. This strongly suggests that the gene encodes an ALDC, and it was therefore named *aldB*. Furthermore, the fragment present in pJIM540 confers ALDC activity in *E. coli* (42). The second ORF encodes a protein of 126 amino acids, which has about 40% identity with several proteins in the data banks. The closest homolog is present upstream of *spoVG* in *B. subtilis* (gi:467437). None of these proteins has a known function, but results presented below suggest that AldR plays a role in BCAA biosynthesis. This gene was therefore named *aldR*.

A potential −10 extended promoter without a −35 box is present in this intergenic region. No canonical promoter was found. The RNA in this region can be folded into a strong secondary structure ($\Delta G°$, −12.4 kcal), whereby the ribosome binding site of *aldB* would be entrapped (Fig. 3).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
NCDO2118	*Lactococcus lactis* subsp. *lactis* natural isolate	NCDO[a]
JIM4173	NCDO2118 Δ(*orf1-aldR*)::*tetM*	This work
JIM4460	NCDO2118 *aldB*::*tetM*	This work
JIM4471	NCDO2118 *aldR*::*tetM*	This work
JIM4571	Spontaneous leucine-resistant mutant from NCDO2118	This work
JIM4572	Spontaneous leucine-resistant mutant from NCDO2118	This work
TG1	*Escherichia coli supE thiD*(*lac-proAB*) *hsdD5* F^+ *traD36 proAB lacIZΔM15*	10
Plasmids		
pVE6004	Thermosensitive pGKV12	31
pBluescript	Amp^r; M13 ori; pBR322 ori	Stratagene
pIL253	Em^r; 4.9 kb	46
pJIM500	18.5-kb *Xba*I fragment of *L. lactis* chromosome in pIL253 containing BCAA operon and *ald* operon	12
pJIM540	1.2-kb *Bam*HI-*Eco*RI fragment of pJIM500 in pBluescript	This work
pJIM557	3.8-kb *Bam*HI-*Xba*I fragment of pJIM500 in pBluescript	This work
pJIM593	3.8-kb *Bam*HI-*Xba*I fragment of pJIM500 in pIL253	This work
pJIM1226	pJIM540 with *tetM* in *Pst*I	This work
pJIM1227	pJIM557 with *tetM* in *Nsi*I	This work
pJIM1230	pJIM1226 with pVE6004 in *Not*I	This work
pJIM1240	pJIM1227 with pVE6004 in *Not*I	This work

[a] NCDO, National Collection of Dairy Organisms, Reading, United Kingdom.

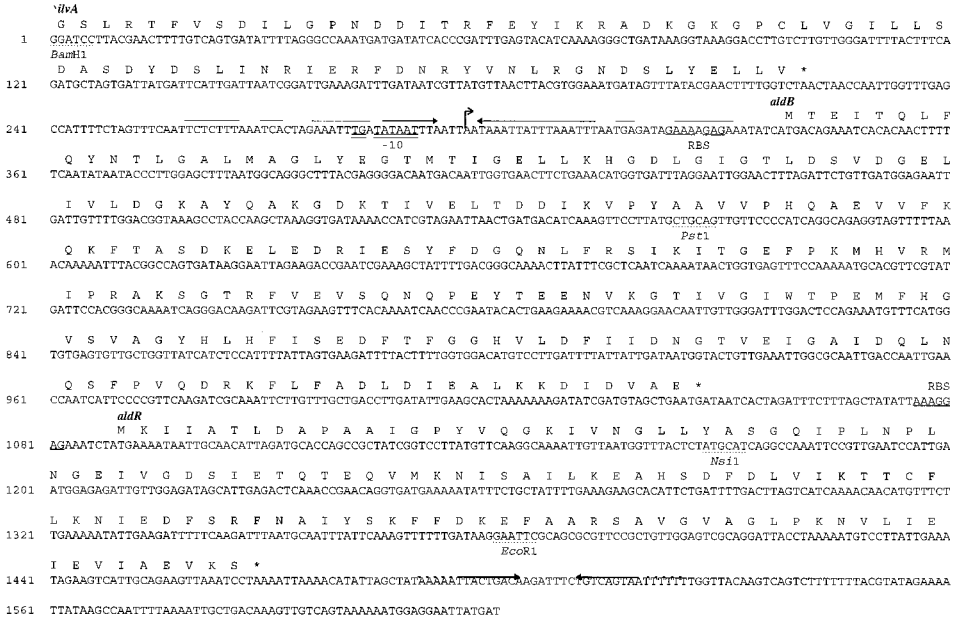

FIG. 3. Nucleotide and deduced amino acid sequence of the *L. lactis* NCDO2118 *ald* region. The numbers on the left refer to nucleotides. Gene names are indicated at the beginning of each amino acid sequence. Translational stop codons and putative ribosome binding sites (RBS) are indicated by asterisks and underlining, respectively. The −10 extended box of the promoter is double underlined. The arrows overlining the ribosome binding site indicate a potential mRNA secondary structure; the boldface arrows at the end of the sequence indicate the putative translational terminator.

Transcriptional analysis of the *ald* genes. To study the transcription of the *ald* genes, cells were grown in liquid CDM supplemented with the three BCAA, washed, and resuspended in minimal medium lacking all BCAA or containing only one of the three BCAA. Cells were held in this medium for 20 min, and their RNA was extracted and analyzed by standard methods. Three transcripts, of 14.5, 7.7, and 1.2 kb, were detected in cells held in the medium lacking BCAA, by using an *ald* probe (Fig. 4). Their size suggests that they extend from promoters P1, P2, and P3, respectively, to the terminator T2 (Fig. 2).

The 5′ end of the 1.2-kb transcript was mapped by primer extension (Fig. 5) to position 296 of the sequence (Fig. 3). This position corresponds to the loop of the potential hairpin structure present between the *ilvA* and *aldB* genes. The potential extended −10 box is located at a proper position upstream of the transcript end.

Interestingly, in resting cells, the two longer transcripts were absent in the presence of isoleucine (Fig. 4) or in rich medium (not shown). The shortest transcript was not affected except in the presence of isoleucine, where its amount was reduced (Fig. 4). Leucine and valine had no important effect on any transcript (Fig. 4). This indicates that isoleucine-dependent transcriptional control contributes to regulating BCAA genes, and to a lesser extent, *aldB.*

The role of ALDC in the BCAA pathway. The role of the *aldB* gene was studied by searching a phenotype of an *aldB* mutant. The mutant was constructed by insertion of the *tetM* gene from Tn*1545* at the beginning of the *aldB* coding frame (codon 75, strain JIM4460 [Fig. 2]). Growth of the parental and that of the mutant strains in various media were compared (Fig. 6). The wild-type strain displayed a relatively long lag phase (6 h) in CDM, followed by exponential growth with the doubling time of about 1.5 h (Fig. 6A, open symbols). It was sensitive to leucine, which prevented significant growth when present at 400 μM (Fig. 6A, closed symbols). Addition of valine at 400 μM to the leucine-containing medium, at the onset of the experiment or after 5 h of incubation, led to immediate exponential growth with a 1.5-h doubling time (Fig. 6A, crosses). This indicates that leucine interferes with valine synthesis in *L. lactis.*

In contrast, the *aldB* mutant strain JIM4460 was resistant to leucine (Fig. 6B, open symbols). The sensitivity was restored by

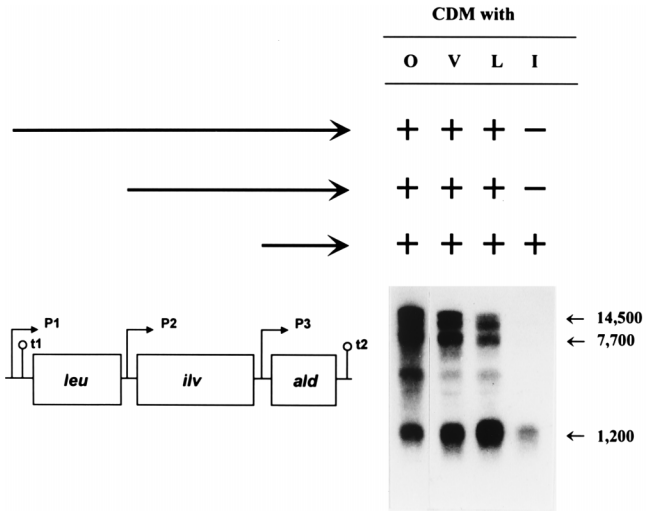

FIG. 4. Map of the transcripts covering the *aldBR* cluster. P1, P2, and P3 refer to transcription promoters, and t1 and t2 refer to transcription terminators. The arrows represent the different RNAs detected by Northern analysis and their sizes in nucleotides. The table on the upper right indicates the presence (+) or the absence (−) of the corresponding transcripts in the cells grown in different CDMs. 0, no BCAA; V, L, and I, CDM with valine, leucine, or isoleucine, respectively, as the only source of BCAA. The autoradiogram of Northern blotting on the right shows the corresponding RNA. The DNA probe is an internal fragment of the *aldB* gene.

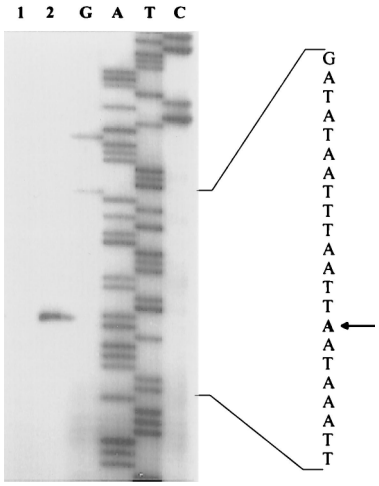

FIG. 5. Primer extension analysis of the *ald* gene transcript using RNA extracted from the mutant strain JIM4173 (lane 1) and the wild-type strain NCDO2118 (lane 2). The lengths of the extension product were determined by electrophoresis on a sequencing gel, with the products of the dideoxy chain termination reaction (lanes G, A, T, and C), in which pJIM540 DNA and the oligonucleotide GCCATTAAAGCTCCAAGGG were used as template and primer, respectively, being used as standards. The sequence corresponding to the strand presented in Fig. 3 is indicated, and the nucleotide corresponding to the transcription start site is highlighted by an arrow.

the introduction of a plasmid carrying an intact *aldB* gene within a 3.8-kb DNA segment (pJIM593 [Fig. 3 and results not shown]). Moreover, two spontaneous Leu^r mutants, JIM4571 and JIM4572, isolated from the parental *L. lactis* strain lost resistance when pIL593 was introduced into them by transformation. These results establish that the leucine sensitivity is due to the *aldB* gene activity.

To evaluate the physiological significance of the leucine control for valine biosynthesis, the MIC of leucine was determined (Fig. 6C). Growth was inhibited at 380 μM leucine and delayed and slowed between 9.5 and 38 μM, whereas it was slightly stimulated at concentrations below 4 μM (Fig. 6C). Thus, 10 μM leucine in the medium seems to be sufficient to exert control of valine synthesis.

Effect of ALDC on pyruvate catabolism. Studies of ALDC in different bacteria have shown that this enzyme is involved in the catabolism of pyruvate. In *L. lactis*, acetoin is produced when the pyruvate pool is high in the cell, when lactate dehydrogenase activity is limited, or during citrate catabolism (26). This suggests that ALDC activity is produced independently of the BCAA pathway. The presence of ALDC in crude extracts of NCDO2118 cells grown in M17 medium, a medium rich in amino acids, confirmed this hypothesis (42). To check whether ALDC activity is encoded exclusively by *aldB*, enzymatic assays were carried out on crude extracts of wild-type and mutant JIM4460 strains grown in the same nutritional conditions. ALDC activity was found in strain NCDO2118 but not in JIM4460. We conclude that *aldB* is the only *L. lactis* gene encoding ALDC and that its product should be involved in pyruvate catabolism, in addition to its role in the BCAA pathway.

To further study phenotypic consequences of the *aldB* mutation for pyruvate catabolism, strain JIM4460 was cultivated in the presence of BCAA under various conditions and compared to the parental strain. In all experiments with glucose or lactose as a carbon source, under aerobiosis or on oxygen-limited growth, growth rates and final concentrations of lactate, formate, acetate, and ethanol were similar in both strains

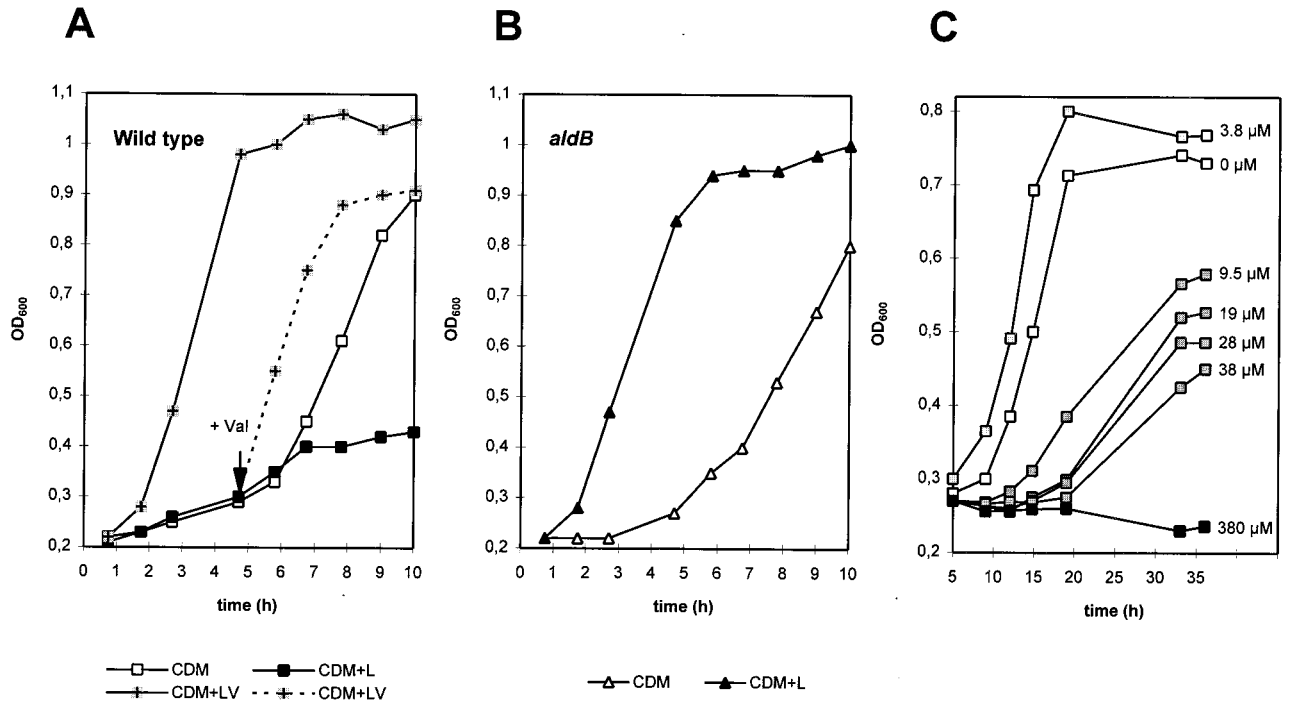

FIG. 6. (A and B) Growth of *L. lactis* NCDO2118 (wild-type strain) (A) and its *aldB* derivative JIM4460 (B) in CDM without leucine, valine, and isoleucine (open symbols); supplemented with 0.4 mM leucine (closed symbols); and with leucine and valine (plus sign in a gray square). The curved dotted line shows the effect of addition of valine to CDM containing leucine at a time corresponding to the arrow. (C) Growth of NCDO2118 cells, washed and resuspended in this medium with the indicated amount of leucine. OD_{600}, optical density at 600 nm.

TABLE 2. Production of AL and of acetoin plus diacetyl[a]

Culture condition	Strain	AL (mM)		Acetoin-diacetyl (mM)		Total (mM)	
		A	B	A	B	A	B
Glucose	Wild type	–	–	0.065	0.058	0.065	0.058
	aldB	0.05	0.015	0.02	0.056	0.07	0.071
Lactose	Wild type	0.01	–	0.3	0.3	0.31	0.3
	aldB	1.1	0.7	0.14	1	1.24	1.7
Lactose plus O_2	Wild type	0.03	–	10	20	10.03	20
	aldB	16	19	1.2	10	17.2	30

[a] Total, sum of the production of the three compounds at the end of exponential phase (A) and after establishment of the stationary phase (B). Cultures were grown with glucose or lactose (10 g/liter) as the source of sugar as indicated. They were maintained anaerobically or in a shaken flask (plus O_2). The A columns correspond to cultures after 6 and 19 h in glucose and lactose, respectively, and the B columns correspond to cultures after 11 and 30 h in glucose and lactose, respectively. Minus signs indicate that the amounts were not measurable by our methods.

(not shown). Acetoin, AL, and diacetyl were produced continuously during cell growth, and the maximum amounts were generally found at the beginning of the stationary phase (Table 2). No significant amount of 2,3-butanediol could be detected in these experiments. Compounds of the acetoin pathway were more abundant on lactose, a slowly catabolized sugar in this strain lacking the PTS^{lac} and tagatose pathway (13), than on glucose, irrespective of the strain. Moreover, this metabolic pathway was strongly stimulated by aerobiosis (Table 2) (3, 10).

AL accumulation during exponential growth was 100-fold higher in the *aldB* mutant than with the wild-type strain, which is consistent with the lack of ALDC activity. Furthermore, AL accumulated to concentrations of 20 mM without provoking a major change in the growth rate, suggesting that it is not toxic at this concentration. It also indicates that AL production is not subject to a strict control mechanism. Final total concentrations of acetoin pathway compounds were similar in the wild-type and the *aldB* strains except when the mutant was grown with anaerobiosis on lactose, in which a fivefold-higher amount of these compounds was observed with the former (Table 2). At the end of exponential growth of the *aldB* mutant, the AL concentration decreased slowly, concomitant with an increase in the total amount of acetoin and diacetyl. This is due to the spontaneous chemical degradation of AL, an unstable compound, to diacetyl and acetoin (44).

Effect of *aldR* inactivation on BCAA biosynthesis. The *aldR* gene was disrupted by insertion of *tetM*, resulting in the strain IL4471. The growth of the *aldR* mutant was measured in CDM with different compositions of BCAA. First, the *aldR* mutant had a growth similar to that of the wild type in CDM with all BCAA and was still inhibited by leucine in the absence of valine (Fig. 7A), which shows that the *aldR* product is not necessary for the expression of ALDC activity. However, in CDM lacking all three BCAA or isoleucine only, the *aldR* mutant growth was slowed down twofold (Fig. 7B). Addition of ketomethylvalerate, the last intermediate of isoleucine biosynthesis (Fig. 1), restored normal growth to the JIM4471 strain. However, addition of ketobutyrate, the first intermediate specific to isoleucine synthesis, did not (not shown). This suggests that the *aldR* product interferes with a middle step of isoleucine synthesis.

DISCUSSION

The *L. lactis* subsp. *lactis* operon, which contains the structural genes for BCAA synthesis, includes two additional genes at the 3′ end. One of these genes, named *aldB*, encodes an

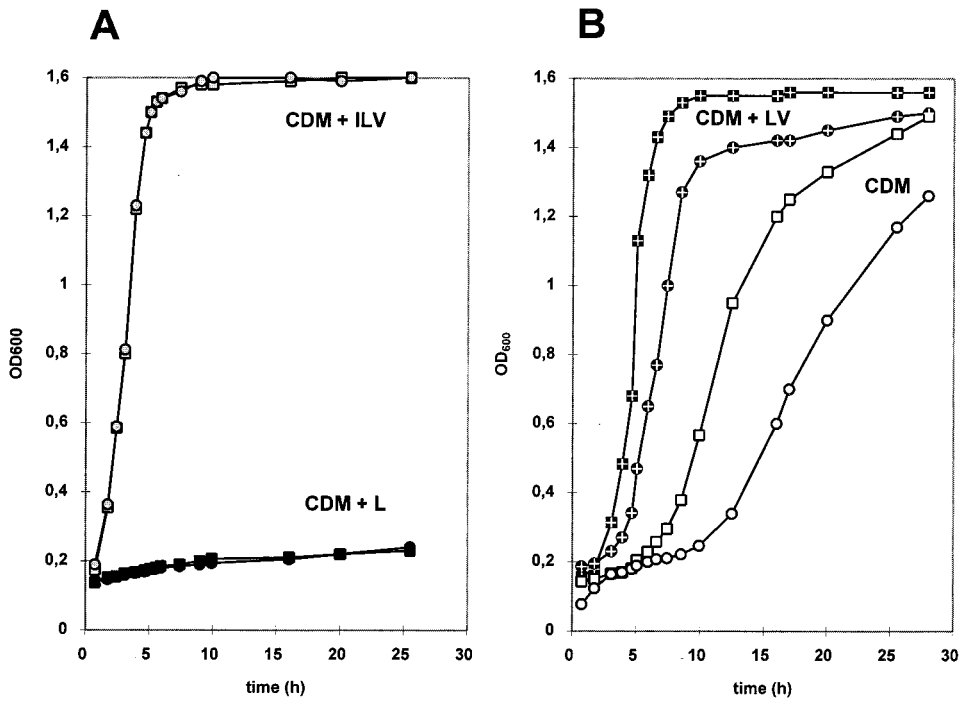

FIG. 7. Effect of isoleucine starvation on the wild type (squares) and the *aldR* mutant (circles). (A) CDM plus isoleucine, leucine, and valine (gray symbols) and CDM plus leucine (closed symbols); (B) CDM (open symbols) and CDM plus leucine and valine (symbols with plus signs).

ALDC, as judged by sequence homology with other ALDC genes, expression in *E. coli* (42), and its effect on *L. lactis* metabolism. The gene does not specify a leader peptide required for protein export, suggesting that the protein is intracellular, as in most bacteria with the exception of *B. brevis* (11). The other gene, *aldR*, encodes a protein which has homology with proteins in the data banks whose function is still unknown. The reduced growth of the *aldR* mutant in the absence of isoleucine suggests that it interferes with the synthesis of this amino acid. However, AldR is not an enzyme directly involved in isoleucine biosynthesis, which requires the *ilvBNCDA* genes clustered upstream of *aldB*. It probably does not regulate the expression of these genes, which are also required for leucine and valine synthesis. If AldR is a protein involved in modulating the specific activity of one or more enzymes, the obvious choice would be IlvA, since it is specific to isoleucine. However, the addition of ketobutyrate does not restore normal growth, suggesting that AldR might be involved in modifying other enzymes for affinity or that it might control the degradation of one of the intermediates. Further work is required to understand the exact function of this protein.

The expression of the *ald* genes was studied by analyzing their transcription. Three transcripts were found to cover the *ald* genes. The two longer transcripts, which extend across the entire BCAA operon and across the *ilv* and *ald* genes, respectively, are presumably initiated at putative promoters detected by sequence analysis (16). Their synthesis is repressed in the presence of isoleucine but not leucine and valine. The third transcript covers only the *ald* genes. It originates at a site which is not preceded by a canonical *L. lactis* vegetative promoter, as no sequence resembling the −35 box is present. However, an extended −10 promoter box (TGNTATAAT) is found at an appropriate distance from the 5′ end of this transcript. In *E. coli*, promoters with such an extension have been shown to be active in the absence of a −35 box. Furthermore, it has been reported that streptococci frequently have these few additional bases upstream of the −10 box (48).

ALDC converts AL, a precursor of leucine and valine, into acetoin and could therefore interfere with the synthesis of these amino acids (Fig. 1). It is intriguing that the *aldB* gene, which specifies this enzyme, is a part of the BCAA operon, which directs the synthesis of leucine, valine, and isoleucine. A possible answer to this apparent contradiction comes from the observation that *aldB* mediates the leucine sensitivity of *L. lactis*. The excess of leucine might signal to the cell that there is no need for the BCAA synthesis and that the metabolic flow should be directed from α-AL towards acetoin rather than towards leucine and valine. Acetoin might then be further reduced to 2,3-butanediol, with concomitant regeneration of NAD^+ from NADH, making NAD^+ available for a variety of metabolic reactions. This system would be fooled by an excess of leucine into emptying the AL pool and therefore preventing the synthesis of valine, which would lead to the observed leucine sensitivity. In members of the family *Enterobacteriaceae*, flux control toward leucine and valine is mediated by valine, which inhibits the enzymes synthesizing AL. The good growth of NCDO2118 in the presence of valine and in the absence of isoleucine suggests that *ilvBN* is not subject to a strong valine retroinhibition in *L. lactis*. The metabolic shunt to acetoin would thus appear to be an alternative control to retroinhibition of the first step of BCAA biosynthesis.

The allosteric properties of the ALDC support this hypothesis, as the purified enzyme is not active in the absence of leucine in vitro (42). Its activity is increased 35-fold when 10 mM leucine is added. In this work, we showed that the control of AL flux to acetoin or valine and leucine seems to be effective in vivo at a level as low as 10 μM extracellular leucine. This concentration is in the range of the apparent affinity constant for transport for leucine in *Lactococcus* spp. (43). For this extracellular concentration, the intracellular concentration of leucine would be around 60 μM in the cell, as deduced from accumulation studies carried out with *L. lactis* membrane vesicles (43). This concentration is higher than the K_m for leucine from the cognate tRNA synthetase, which was determined to be 5 to 8 μM in several bacteria (1, 27, 47). Thus the maintenance of an intracellular pool of 60 μM leucine should ensure normal growth.

Catabolism of pyruvate into 2,3-butanediol was studied in detail with *Bacillus*, *Aerobacter aerogenes*, and *Klebsiella* (7, 29, 37, 46), which possess two different enzymes, AHAS, the *ilvBN* product, and AL synthase, able to synthesize AL for BCAA biosynthesis and the butanediol pathway, respectively (21, 25). In these bacteria, the genes encoding AL synthase and ALDC are organized in an operon and transcribed under the control of an activator responding to acetate (25). In *Aerobacter*, half of the pyruvate is used for 2,3-butanediol production upon induction of the system. The channeling of pyruvate to 2,3-butanediol production may assist internal pH maintenance by changing the metabolism from acid production to the formation of neutral compounds. In addition, it may participate in the regulation of the $NADH/NAD^+$ ratio by the reversible conversion of acetoin and 2,3-butanediol. However, the kinetic properties of the enzymes participating in this pathway in these bacteria differ markedly from those of *L. lactis*. First, the affinity of *L. lactis* AL synthase for pyruvate is very low, with a K_m of 50 mM (50) compared with 6 and 13 mM for *A. aerogenes* and *B. subtilis* enzymes (18, 22), respectively, and 8 mM for AHAS of *L. lactis* (4). This limits the flux toward AL. The butanediol pathway is used mainly when lactate dehydrogenase activity is limited, at low fructose 1,6-diphosphate (55) and NADH (13) concentrations, during sugar-limited growth and aerobiosis, respectively. Interestingly, a number of lactic acid bacteria, such as *L. lactis* subsp. *lactis* bv. diacetylactis (22), *Leuconostoc lactis* (9), *Lactobacillus lactis* (30), and *Lactobacillus plantarum* (56), generate acetoin from exogenous citrate, which is taken up by a plasmid-encoded citrate permease. In *L. lactis*, the degradation of citrate presumably leads to an excess of pyruvate, which is converted subsequently into AL. In lactic acid bacteria, citrate fermentation could be coupled to the generation of metabolic energy by exchange of the products through the membrane (26, 52). However, with the exception of *L. lactis*, AL synthases from the lactic acid bacteria have a high affinity for pyruvate and ALDC is not controlled by BCAA. This suggests that AL metabolism in *L. lactis* is rather particular. No residual ALDC activity was found in *aldB* mutants of our model strain NCDO2118 or other *L. lactis* subsp. *lactis* strains able to utilize citrate (18). This suggests that these bacteria use the same enzyme to control BCAA biosynthesis and the acetoin catabolic pathway. This metabolism is not controlled by AL, as the strains deficient in ALDC accumulate large amounts of AL during aerobiosis or sugar limitation without effect on their growth. The absence of AL feedback control strengthens the role of *aldB* in pyruvate catabolism, allowing bacteria to avoid AL accumulation and possible production of diacetyl, an AL derivative which might be toxic for the cell (28).

The dual role of ALDC in *L. lactis* is unique. The *aldB* gene, encoding ALDC, seems to be constitutively expressed from the promoter P3, leading to a constant presence of the enzyme in the growing cell. This could be consistent with the constitutive expression of *alsS*, encoding catabolic AL synthase, in this bacterium (36). When the biosynthetic genes *leu* and *ilv* are

expressed, *aldB* would be additionally transcribed from promoters P1 and P2. This could increase ALDC expression to a level which could prevent leucine and valine biosynthesis in the absence of a strong allosteric control limiting AL catabolism. We speculate that, in this case, *aldB* expression might be under a supplementary control. The potential secondary structure encompassing the *aldB* ribosome binding site could be responsible for the control of the translation level of this gene.

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